IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	) Art Unit: 1612
STRÖMBLAD et al	) Examiner: PACKARD, Benjamin J
Appln. No.: 10/550,516	) Alexandria, VIRGINIA
Filed: March 24, 2004 371(c) dated: May 26, 2006	) Confirmation No. 4509
For: Pharmaceutical use of…	) ATTY.'S DOCKET # P07900US01/BAS

DECLARATION UNDER 37 CFR 1.132

I, Staffan Strömblad, hereby solemnly declare as follows.

I was awarded a PhD degree in Experimental Pathology from the Karolinska Institutet, Sweden in 1992. In 2000, I was appointed Associate professor at Karolinska Institutet. Attached is a shortened form of my CV which is made a part hereof.

I am a co-inventor of the above-identified U.S. patent application. I have read and understand the subject matter of the Office Action of January 9, 2009, including the cited references. In my opinion, the Examiner's allegations are incorrect.

In the Office Action, the Examiner has taken the position that the specification does not reasonably provide enablement for inhibiting undesired angiogenesis. However, it is my

opinion that the application does provide enablement also for inhibiting undesired angiogenesis. The reason is the following: First of all, the application describes experiments convincingly showing that:

- wild type p53 (wtp53) may be present in the cell in an inactive conformation *despite* being non-mutated;
- the inactivation of wtp53 is mediated by the integrin $\alpha_v\beta_3$; and
- the active conformation of wtp53 in the presence of integrin $\alpha_v\beta_3$ may be restored by the inventive compounds.

In two papers, viz. Strömblad et al., J. Clin. Invest. 98:426-433 (1996); and Strömblad et al., J. Biol. Chem. 277: 13371-13374 (2002), referred to at page 7 the present application, the involvement of p53 and integrin $\alpha_v\beta_3$, respectively, in angiogenesis is discussed. These papers together teach that in neovascularization of wildtype animals, α_v-integrins, such as $\alpha_v\beta_3$, are activated in the proliferative endothelial cells and that this activation leads to a suppression of the activity of p53. Furthermore, it is shown that when the integrins are blocked, by use of integrin antagonists, endothelial p53 activity is restored and the vascular cells undergo apoptosis. It also is shown, in p53 null mice, that angiogenesis is refractory to inhibition of α_v-integrins, thus functionally

linking in vivo the $\alpha_v\beta_3$-mediated inactivation of p53 in vascular cells to their capacity to undergo angiogenesis.

It appears that the cell survival of both malignant melanoma cells and endothelial cells undergoing angiogenesis is under the influence of integrin $\alpha_v\beta_3$, suppressing the activity of wtp53.

As pointed out herein above, in the present application, the inventors have shown that the apoptosis-inducing activity of wtp53, lost in the presence of $\alpha_v\beta_3$, may be restored by treatment with the inventive compounds. In view of the teachings of the two prior art documents referred to herein above, it is my firm belief that this same principle may also be applied in endothelial cells undergoing angiogenesis under the influence of $\alpha_v\beta_3$ and the inventive compounds therefore also may be applied in a method of treating undesired angiogenesis.

The Examiner furthermore has taken the position that the specification does not reasonably provide enablement over the whole scope of compounds as defined in claim 1 of the application.

However, it is my belief that the specification does provide enablement over the whole scope of compounds as defined in present claim 1.

First of all, in vitro and in vivo experiments have been performed not only on the compound referred to as Prima-1, but also on the compound 2-(hydroxymethyl)-2-methoxymethyl)-quinuclidin-2-one, of structural formula:

herein below referred to as Prima-1^{MET}.

By these experiments, it was shown that Prima-1^{MET} is able to rescue unfolded wtp53, induce melanoma cell apoptosis and suppress melanoma growth in vivo in a wtp53-dependent manner.

The details of the experiments are described in Appendix 1, whereas Appendix 2 contains Figures 1-4 as referred to herein below.

In the experiments, the p53 conformation was examined by two-site ELISA in wtp53-carrying, integrin αvβ3-positive M21, AA and FM88 human melanoma cells. In all cell lines, p53 dis-

played an active (PAb1620+) conformation in regular two-dimensional (2D) culture (d 0) (Figs. 1a and 2), as expected for wtp53. Surprisingly, wtp53 conformation was switched to an inactive (Pab240+) conformation (Figs. 1a and 2), characteristic for partially unfolded mutant p53 proteins, in all cell lines in a three-dimensional (3D)-collagen gel mimicking the pathophysiological dermal microenvironment of malignant melanoma growth in vivo. Notably, this switch was integrin αv-dependent, as evident from comparison of αv-positive M21 cells and M21L cells, an M21 subpopulation lacking αv-integrins9. In regular 2D-culture (d 0), the active p53 conformation was dominant in both M21 (αv+) and M21L (αv-) cells (Fig. 1a). However, after 5-7 d in 3D-collagen, a high proportion of inactive p53 conformation was detected in M21 cells (αv+), whereas the active p53 conformation was still dominating in M21L (αv-) cells (Fig. 1a). This suggests that integrin αv induces partial unfolding of wtp53 in 3D-collagen. Importantly, the unfolding of wtp53 in αv-expressing M21 cells correlates with loss of p53 function: as previously demonstrated, M21 (αv+) cells have low p53 activity and survived while αv-negative M21L cells die from p53-dependent apoptosis in 3D-collagen as well as in vivo. Thus, induction of an inactive conformation of wtp53 mediated by integrin αv is critical for melanoma cell survival in a 3D-environment.

It was found that Prima-1^{MET} markedly shifted the balance of wtp53 conformation from an inactive to the active p53 conformation in the three distinct melanoma cell lines M21, AA and FM88 within 3D-collagen (Fig. 1b). Prima-1^{MET}-mediated conversion of unfolded wtp53 into an active conformation was accompanied by induction of the p53 transcriptional targets PUMA and Apaf1 (Fig. 3), suggesting functional activation of p53. Moreover, Prima-1^{MET} induced melanoma cell apoptosis in 3D-collagen in a wtp53-dependent manner (Figs. 1c and 3). M21 cells were resistant to a high dose (400 μM) of the anti-tumor drug 5-Fluorouracil (data not shown). Thus, the effect of Prima-1^{MET} appears to be different from that of a genotoxic agent known to act via wtp53. Importantly, Prima-1^{MET} treatment substantially suppressed in vivo tumor growth of M21 but not M21-p53siRNA s.c. xenograft tumors in mice (Figs. 1d, 4a and 4b), suggesting that the tumor suppressor effect of Prima-1^{MET} on melanoma cells in vivo is wtp53-dependent. In addition, Prima-1^{MET} significantly inhibited FM88 xenograft tumor growth (Fig. 4c). Thus, targeting of unfolded wtp53 by Prima-1^{MET} had a potent tumor inhibitory effect in different human melanoma xenograft models.

As is known, Prima-1 can revert *mutant* p53 into an active form, and it also has been previously shown that Prima-1^{MET} too

has this faculty; cf. e.g. Bykov, V. J. et al. Nat Med 8, 282-288 (2002).

Thus, it appears most likely that compounds capable of reverting mutant p53 into an active form also have the faculty of reverting wtp53 into an active form.

US application 10,590,054 (publication No. US 2007-0142370) (herein below referred to as US '054) describes and claims compounds, falling within the scope of claim 1 of the present application, for the treatment of diseases associated with mutant p53 or, more generally, a malfunctioning p53 signalling pathway.

Thus, US '054 describes tests showing the antiproliferative and apoptosis inducing effects of a fair number of compounds within the scope of claim 1 of the present application, using a human H1299-His175 lung carcinoma cell line that carries a tetracycline-regulated mutant p53 construct; cf. Table 1 of US '054.

In analogy with the compounds Prima-1 and Prima-1^{MET} it therefore is my firm belief that also the compounds tested according to US '054 will have the required activity of reverting

wtp53 into an active form, thereby being useful in the treatment malignant melanoma and undesired angiogenesis.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: _____

Professor Staffan Strömblad, PhD

Date: May 8th, 2009

Curriculum Vitae of Professor Staffan Strömblad, PhD

A. Professional preparation

1988	B.Sc. Biochemistry & Microbiology, University of Stockholm.
1992	Ph.D. Karolinska Institutet. Area: Experimental Pathology. Thesis entitled: "The epidermal growth factor receptor system in rat liver growth and carcinogenesis".
1994-1996	Post-doctoral training, The Scripps Research Institute, La Jolla, CA, in the laboratory of Dr. David A. Cheresh. Area: Integrins and angiogenesis.

B. Appointments

2003-2009	Senior scientist award from the Swedish Research Council
2000	Associate professor (docent), Karolinska Institutet.
1997	Principal investigator and Assistant professor, Karolinska Institutet.
1997-2003	Cancer researcher position by The Swedish Cancer Society.
1994-1996	Research associate, The Scripps Research Institute (3 yrs).
1993	Post-doctoral researcher, Karolinska Institutet.
1988-1992	Post-graduate student, Karolinska Institutet.

C. Graduated students and post-doctoral fellows

Wenjie Bao, PhD 2005-06-17; Annica Gad, PhD 2005-08-26. Totally eleven different post-doctoral fellows have worked under my supervision 1998-2009. I am presently main supervisor for 5 Post-docs and 4 PhD students, and co-supervisor of 4 PhD students.

D. Fellowships and awards

2007	Erik K. Fernström's prize for young investigators, University of Lund, Sweden
2004	Awarded the KI-Cancer strategic grant
2003	Senior scientist Award position in Clinical Molecular Biology from the Swedish Research Council
1996	The Wenner-Gren Foundation post-doctoral fellowship
1994-95	The Swedish Cancer Society post-doctoral fellowship

E. Commissions of trust

- Deputy chairman of evaluation committee (study section) at the Swedish Cancer Society 2008- .
- Member of an international evaluation board for Academy of Finland; yearly evaluations 2007- .
- Member of evaluation committee (study section) at the Swedish Cancer Society 2006 -2008
- Board member of the KI-Cancer network 2004-2006.
- Chairman of the review committee for the Robert Lundberg memorial foundation 2002-2005.
- Member of the department/division boards during various periods, 1988-1992 and 1997-2004.
- A long number of commissions of trust within academia as a student, including as vice president of the Federation of the Student Unions of Stockholm (SSCO) 1987-88, board member of the Stockholm Student Housing Foundation (SSSB) 1987-91 and of the Stockholm Students Sports Federation 1986-91, board member of the Stockholm University Student Union 1987-88 and Chairman of the Student health care organization in Stockholm (Studenthälsan)1987-88.
- Faculty opponent at two PhD dissertations (Tallin Technical University, Estonia, 2000; Turku University, Finland, 2005) and one licenciate dissertation (Stockholm University, Sweden, 2005).
- Assignments as ad hoc reviewer for the following journals; Artheroscl. Thrombosis Vascul. Biol.; Am. J. Pathol.; Blood; Cancer Lett.; Cell; Exp. Cell Res.; FASEB J.; J. Cell Biol.; J. Cell Sci.; J. Clin. Invest.; Mol. Biol. Cell; Proc. Natl. Acad. Sci. USA; Trends Cell Biol.; Trends Mol. Med.

F. Science and society, Entrepreneurial achievements

- Co-founder of two start-up biotech companies within the Oncology field; Angitia AB and Aprea AB, both in operation.
- Author of two patent applications.

G. Impact/citations

My scientific papers have been cited a total of more than 2900 times (ISI, March, 2009).

H. International invitations and conferences

- Invited speaker at approximately fifteen international conferences/symposia, including as a Keynote speaker at the annual meeting of the Dutch working party for tumor biology, Lunteren, The Netherlands, May 6-7, 1996.
- Invited speaker at approximately twenty foreign institutes, universities, etc.
- Organizer of an international symposium on Angiogenesis at Nobel Forum, Karolinska Institutet, September 1997.
- Organizer of a Minisymposium on Fluorescent live cell imaging at Karolinska Institutet, February 2004.

I. Five selected publications

1. **Strömblad, S.**, Becker, J.C., Yebra, M., Brooks, P.C. & Cheresh, D.A. Suppression of p53 activity and $p21^{WAF1/CIP1}$ expression by vascular cell integrin $\alpha_v\beta_3$ during angiogenesis. **J. Clin. Invest.** 98, 426-433 (1996).

2. Zhang, H., Li, Z., Viklund, E-K. **& Strömblad, S.** p21-activated kinase 4 interacts with integrin avb5 and regulates avb5-mediated cell migration. **J. Cell Biol**. 158, 1287-1297 (2002).

3. Zhang, H., Berg, J., Li, Z., Wang, Y., Lång, P., Sousa, A.D., Bhaskar, A., Cheney, R.C. **& Strömblad, S.** Myosin-X provides a motor-based link between integrins and the cytoskeleton. **Nature Cell Biol.** 6, 523-531 (2004).

4. Bao, W. **& Strömblad, S.** Integrin αv-mediated inactivation of p53 controls a MEK-1-dependent melanoma cell survival pathway in three-dimensional collagen. **J. Cell Biol**. 167, 745-756. (2004).

5. Thullberg, M., Gad, A., LeGuyader, S. **& Strömblad, S.** Oncogenic H-Ras V12 promotes anchorage-independent cytokinesis. **Proc Natl Acad Sci USA**. 104, 20338-20343 (2007).

Appendix 1: Experimental methods

Cell culture and stable transfection

Human melanoma AA, FM88, M21 (αv+) and M21L (αv-) cells were kept in culture of RPMI-1640 with 5 % FCS and applied into a three-dimensional collagen (3D-collagen) gel model as described[1,2]. PRIMA-1[MET], a methylated analogue of PRIMA-1 was used in the range of 20-100 μM, and 5-Flurouracil (Sigma) used at 60 μM. These compounds were mixed into the collagen before gel polymerization. For stable transfections, M21 cells were co-transfected with 10 μg of pSUPER-p53[3] and 0.1 μg of pCI-neo plasmid using Lipofectamine 2000 (Invitrogen Life Technologies). The selection of positive clones was performed as previously described[2].

Flow cytometry and ELISA

Cell surface expression of integrin αvβ3 in AA, FM88 and M21 cells was detected by flow cytometry as described[2]. The p53 conformation was analyzed by an two-site ELISA as described[4]. Briefly, p53 was captured from lysates using antibodies immobilized on ELISA plates: conformation-specific mabs PAb1620 (Oncogene) or PAb240 (Oncogene) to recognize a folded and an unfolded conformation of p53, respectively, and non-conformational anti-p53 mab DO-1 (Santa Cruz, Biotechnology, Inc.) for total p53 protein, used as an internal reference. Polyclonal anti-p53 antibody FL393 (Santa Cruz) was used to detect p53 captured by primary antibodies, followed by application of anti-rabbit secondary antibody conjugated with horse-reddish peroxidase (Vector). The ratio of PAb1620 to PAb240 reactivity was calculated at each time point to determine the relative changes in p53 conformation and to facilitate statistical evaluations.

Immunoblotting

Cell lysates, protein determination and immunoblotting were carried out as previously described[5]. The following primary antibodies were used: anti-p53 mab (DO1) from Santa Cruz; anti-PUMA pab (Ab-1) from Oncogene; anti-Apaf1 mab (2E12) from Alexis; Anti-actin mab (JLA 20) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Detection of apoptosis

Apoptosis was analyzed by Annexin-V staining followed by flow cytometry as described[2]. In certain cases, an inverted phase contrast microscope was used for quantification of cells with

apoptotic membrane blebbing morphology within at least 40 randomly chosen sight areas. At least 600 cells were counted for each condition. The morphological quantifications were similar to the results obtained by staining of apoptotic cells using Apopercentage dye (APOPercentage™ Assay, Biocolor Ltd) (data not shown).

Animal experiments

All animal studies were approved by the Stockholm South animal ethics committee and the animal care was in accordance with institutional guidelines. Female C57BL6 nude mice (6-8 weeks old) (M&B, Danmark) were inoculated with melanoma cells ($1.5\text{-}2 \times 10^6$) s.c. at the flank at the back of the mice. After 6 d, formed tumors were visible in most mice and PRIMA-1^{MET} (100 mg/kgXd) was daily injected i.p. for 6 d. The tumor size was measured every other day with calipers and tumor volumes were calculated by the formula 0.52 X Length X Width X Width. The wet weight of dissected tumors was analyzed at the end of experiments.

Statistical analysis

An unpaired two-tailed t-test was used for statistical analysis in the study using the Microsoft Excel software.

References

1. Montgomery, A. M., Reisfeld, R. A. & Cheresh, D. A. Proc Natl Acad Sci U S A **91**, 8856-60 (1994).

2. Bao, W. & Strömblad, S. J Cell Biol **167**, 745-56 (2004).

3. Brummelkamp, TR., Bernards, R. & Agami, R. Science 296, 550-553.

4. Bykov, V. J. et al. Nat Med **8**, 282-8 (2002).

5. Bao, W. & Strömblad, S. Biol Proced Online **4**, 81-87 (2002).

Appendix 2: Figures 1-4

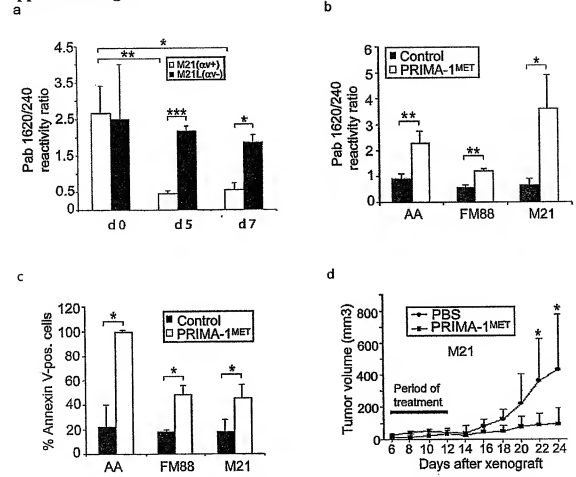

Figure 1. Integrin αv enforces an unfolded, inactive wtp53 conformation in melanoma cells, while PRIMA-1^{MET} restores a folded, active wtp53 conformation, triggers apoptosis and suppresses melanoma xenograft growth in mice. **(a)** p53 conformation was analyzed in M21 (αv+) and M21L (αv-) cells in 2D-culture (d 0) and after 5-7 d in 3D-collagen. The ratio between PAb1620 and PAb240 epitope reactivity was compared in 2D (d 0) to that in 3D (± S.D.; n=3), and in M21 (αv+) cells to that in M21L (αv-) cells in 3D-collagen (± S.D.; n=3). **(b)** Effect of PRIMA-1^{MET} (100 μM) on the PAb1620/PAb240 reactivity ratio after 5 d exposure in 3D-collagen of AA, FM88 and M21 human melanoma cells (± S.D.; n=3). **(c)** Fraction of Annexin-V positive cells after treatment of AA, FM88 and M21 cells with PRIMA-1^{MET} (80 μM) or DMSO control for 36 h within 3D-collagen (± S.D.; n=3). **(d)** Effect of PRIMA-1^{MET} on the growth of M21 cells (wt p53) injected s.c. in C57/BL mice. The line graph shows the mean tumor volumes over time (n=7). Statistics in Fig. 1: $* - p < 0.05$, $** - p < 0.01$, $*** - p < 0.001$ using an unpaired two tailed *t*-test.

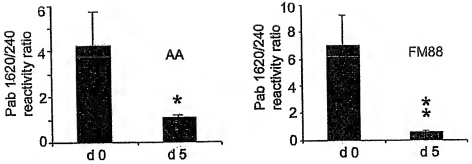

Figure 2. Induction of unfolded wtp53 in melanoma cells in 3D-collagen. The epitope reactivity ratio of PAb1620 (folded p53) and PAb240 (unfolded p53) was compared in wtp53-carrying AA and FM88 human melanoma cells from 2D tissue culture (d 0) and after 5 d in 3D-collagen (± S.D.; n=3; * – $p < 0.05$; ** – $p < 0.01$ using an unpaired two-tailed t-test).

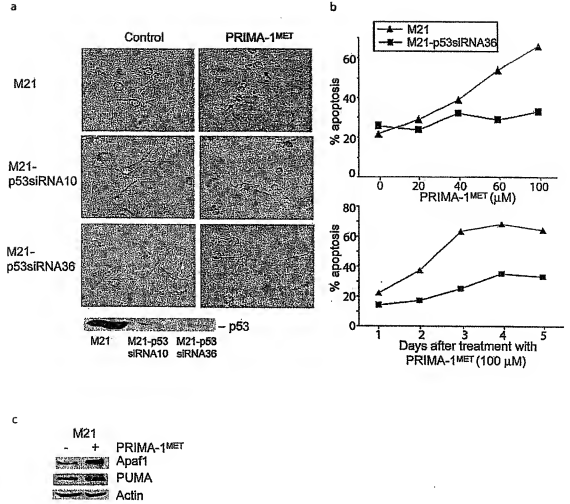

Figure 3. PRIMA-1^{MET} triggers wt p53-dependent melanoma cell apoptosis in 3D-collagen. **(a)** Microscopic images show cells with apoptotic membrane blebbing morphology (arrows) after treatment of M21 and M21-p53siRNA cells with PRIMA-1^{MET} (80 μM) or DMSO control within 3D-collagen. The immunoblot (below) shows p53 protein levels in the cells used. **(b)** Line graphs display: quantifications of membrane blebbing apoptotic cells within 3D-collagen. Upper: PRIMA-1^{MET} treatment for 5 d at different doses of M21 and M21-p53siRNA36 cells. Lower: Time-course of apoptosis after treatment with PRIMA-1^{MET} (100 μM). The displayed results are representative among three independent experiments. **(c)** Protein levels of PUMA, Apaf1 and actin were assessed after PRIMA-1^{MET} (80 μM) treatment for 24 h in 3D-collagen. The displayed results are representative among three independent experiments.

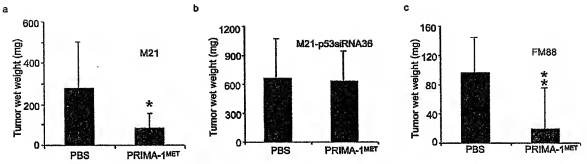

Figure 4. PRIMA-1^{MET} suppresses melanoma tumor growth in a wtp53-dependent manner. M21 (wt p53), M21-p53siRNA (p53 knocked-down) and FM88 (wtp53) human melanoma cells were inoculated s.c. at the back of C57/BL nude mice. The graphs show mean tumor wet weights of dissected tumors of M21 (a), M21-p53siRNA (b), and FM88 (c) after treatment with PRIMA-1^{MET} (100mg/kgẊd) or PBS control. n=6-8; $* - p< 0.05$, $** - p< 0.01$, as compared to PBS control using an unpaired two-tailed t-test.